Docket No.: 30187/41217
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Martin Kintrup et al.

Application No.: 10/533,618 | Confirmation No.: 1749

Filed: February 6, 2006 | Art Unit: 1645

For: MEANS AND METHODS FOR DIAGNOSING A TREPONEMA INFECTION | Examiner: B. J. Gangle

DECLARATION UNDER 37 C.F.R. § 1.132

MS RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

I, Martin Kintrup, Ph.D., declare that:

1. I am a co-inventor named on United States Patent Application No. 10/533,618. I am currently employed as Director Sales and Marketing at Viramed Biotech AG, Behringstrasse 11, 82152 Planegg, Germany, assignee of the instant application. I served as Director of Research and Development at Viramed Biotech AG between 2001 and 2004. The Treponema + VDRL ViraBlot® Tests were developed with my direction. I also developed the following test systems for human diagnostics: EBV ViraStripe® (1999), *Bordetella pertussis* ViraBlot® (2000), ENA ViraStripe® (2000), Leber ViraStripe® (2002), Treponema + VDRL ViraBlot® (2002), Bordetella pertussis ViraStripe® (2003).

2. I studied chemistry and my diploma thesis and dissertation were on topics relating to biophysics, biochemistry and molecular biology, resulting in the following publications:

* Scholz O, Kintrup M, Reich M, Hillen W., J Mol Biol. 2001 Jul 27;310(5):979-86.

* Scholz O, Schubert P, Kintrup M, Hillen W., Biochemistry. 2000 Sep 5;39(35):10914-20.

* Kunz M, Kintrup M, Hillen W, Schneider S., Photochem Photobiol. 2000 Jul;72(1):35-48.

* Vergani B, Kintrup M, Hillen W, Lami H, Piémont E, Bombarda E, Alberti P, Doglia SM, Chabbert M., Biochemistry. 2000 Mar 14;39(10):2759-68.

* Kintrup M, Schubert P, Kunz M, Chabbert M, Alberti P, Bombarda E, Schneider S, Hillen W., Eur J Biochem. 2000 Feb;267(3):821-9.

* Alberti P, Bombarda E, Kintrup M, Hillen W, Lami H, Piémont E, Doglia SM, Chabbert M., Arch Biochem Biophys. 1997 Oct 15;346(2):230-40.

* Schmiedel D, Kintrup M, Küster E, Hillen W., Mol Microbiol. 1997 Mar;23(5):1053-62.

* Lederer T, Kintrup M, Takahashi M, Sum PE, Ellestad GA, Hillen W., Biochemistry. 1996 Jun 11;35(23):7439-46.

3. My expertise in the field of diagnostics is documented by many conference contributions, including, for example:

* International Potsdam Symposium on Tick-Borne Diseases, Jena, Germany, March 2005: "Improvement of Serodiagnosis In Early Lyme Borreliosis By European Lysate Blot With VlsE" by Heidelore Hofmann, Ingrid Lorenz, Eva Magyar, Vera Helbl, Heike Thüring-Nahler, Elke Witte, Martin Kintrup.

* International Jena Symposium on Tick-Borne Diseases, Jena, Germany, March 2007: "Densitometric Analysis of Immunoblot Band Intensities from Patients with Early Lyme Borreliosis for Treatment Follow-up" by Heidelore Hofmann, Ingrid Lorenz, Eva Magyar, Vera Helbl, Elke Witte, Martin Kintrup.

4. I have experience and knowledge in the field of immobilizing proteins and antigens on solid carriers, e.g., nitrocellulose membranes. My research projects involve microdispensing technologies, allowing formation of liquid drops in the nanoliter range that are transferred to nitrocellulose membranes. The immobilized proteins/antigens can be

incubated with a biological sample, which may contain antigen specific antibodies. The presence of antigen specific antibodies in a sample is verified by visualizing antigen-antibody complexes. My special expertise includes an understanding of how chemical and physical conditions influence immunoassays, particularly the sensitivity and specificity properties of a test system.

5. Physical parameters that influence antigen-antibody test systems include, e.g., rheology of the liquid, air humidity, temperature, dispensing speed and volume, wetting and binding kinetics of the carrier. Exemplary chemical parameters include, e.g., pH and ion strength of the liquid (antigen containing) phase, as well as additives such as polymers, sugars, and surfactants. Finishing treatment of the antigen-loaded membranes, i.e., fixation and blocking of unsaturated binding sites, are important factors for the test system as well. Adequate sensitivity and specificity are essential for diagnostic test systems in the field of enzyme immunoassays.

6. When proteins/antigens are transferred onto a carrier, e.g., a nitrocellulose membrane, the proteins/antigens must remain bound to the carrier material during each subsequent step required to create a carrier suitable for diagnostic assay. One such step comprises treating the carrier to block free binding sites on carrier material and to reduce non-specific binding to immobilized antigens. Free binding sites on the carrier material must be treated with reagents to prevent adsorption of components from the biological sample (e.g., serum) or from other test kit reagents. Otherwise, the carrier would show high background reaction that would influence the final test result. In addition, undefined components from serum or reagents could bind non-specifically to antigens, thereby reducing the specific reactivity of the antigen.

7. In 2002, there was no test system commercially available or described in the literature that was able to detect anti-cardiolipin (e.g., VDRL) reagin antibodies simultaneously with anti-Treponema antibodies in one assay, i.e., within one reaction chamber or on one carrier. This is because the antigens have different chemical natures:

cardiolipin lipids are small hydrophobic molecules, whereas Treponema proteins are larger molecules that are hydrophilic in most cases (depending on buffer conditions). Detergents are often used to reduce unspecific binding to the protein antigens to achieve the sensitivity and specificity required for diagnostic tests. Sambri et al., *Clinical and Diagnostic Laboratory Immunology*, *8*(3), 534-539 (2001), for example, discloses incubating Treponema antigen-containing test strips with TWEEN® 20. However, it was not believed that detergents could be used with lipid antigens. Lipids generally are solubilized by detergents and cannot be immobilized on a carrier surface in the presence of detergent. In the field of Treponema diagnostics, this phenomena was confirmed by many scientists and reviewed in, e.g., International Patent Publication WO 91/10138 at page 2, lines 2-24:

> Numerous investigators have tried to improve reagin tests by incorporating the cardiolipin antigen into ELISA-type procedures. These ELISA assays . . . are not as specific or sensitive as the RPR or VDRL tests. For example, ADI Diagnostics claim a specificity of 95.4% and sensitivity of 82.2% compared to the VDRL test.
>
> These ELISA types of assays, however, do not perform satisfactorily in the presence of detergents such as Nonidet P-40 or Tween 20. The use of detergents in EIA or ELISA reduces reagent nonspecific binding and hence greatly enhances the sensitivity and specificity of the assay. Since the detergent cannot be used in these previously documented assay (CARD, PC and CHOL are presumably removed from the solid phase), they are not very sensitive or specific.

8. Initial experiments by my research team to create a single carrier comprising both cardiolipin and *Treponema*-specific antigen using detergent failed. We attempted to generate a nitrocellulose carrier comprising immobilized cardiolipin and immobilized 47 kD *Treponema* antigen employing the same detergent concentration disclosed in the Sambri reference, 0.05% TWEEN® 20, which is a buffer system used to prevent non-specific binding in immunoassays. The conditions allowed subsequent detection of antibodies specific for *Treponema* protein antigens on the carrier. However, exposure of the carrier to detergent solubilized cardiolipin and released the VDRL antigen from the test strip. As a result, VDRL reactivity could not be detected. Appendix A is a reproduction of five immunodiagnostic test strips that were generated using 0.05% TWEEN® 20 and that failed to detect VDRL reactivity. A key containing handwritten notes identifying the

antigens and antigen concentrations applied to the test strip is depicted at the top of the reproduction. The key demonstrates the position of the various antigen bands (reading from left to right along the key): protein A (control), IgG (control), cut-off control, VDRL (1:2 dilution), VDRL (1:4 dilution), VDRL (1:16 dilution), VDRL (1:32 dilution), VDRL (1:128 dilution), 47 kD *Treponena* antigen (75 μg/ml), 44.5 kD *Treponema* antigen (3.5 μg/ml), 17 kD *Treponema* antigen (9.5 μg/ml), 15 kD *Treponema* antigen (18.75 μg/ml). VDRL dilutions were generated from an ethanolic solution of 0.3% cardiolipin, 0.9% cholesterol, and 0.18%-0.20% lecithin. The five test strips were developed using positive or negative samples: VM 546 (serum previously tested positive in conventional VDRL test and Treponema antigen tests for IgG and IgM), VM 1332 (serum previously tested negative in VDRL test and Treponema antigen tests for IgG and IgM), BD VDRL + (positive control, confirmed in VDRL test), BD VDRL +/- (control, being equivocal in conventional VDRL test), BD VDRL - (negative control, confirmed in VDRL test). Test strips were exposed to 20 μl of sample in 1.5 ml of a washing buffer containing 0.05% TWEEN® 20 (similar to that described in the Sambri reference), and subsequently washed with the buffer to remove unbound sample components. After washing, test strips were incubated with an anti-human IgG antibody conjugated to alkaline phosphatase (secondary antibody) and washed with buffer. A chromogen/substrate was applied to form a colored precipitate at bands where secondary antibody was bound. Colored precipitate, observed as bands on the test strip, indicates that the tested sample contained antibodies specific for the bound antigen. Regardless of the samples being pre-characterized as containing or lacking VDRL antibodies, no VDRL antigen bands were visualized on the test strips. Developing the test strips using the detergent concentration taught in the Sambri reference rendered the strips incapable of detecting anti-VDRL antibodies.

9. WO 91/10138 teaches elaborate techniques for immobilizing lipids to withstand treatment with detergent at page 4, line 7, to page 5, line 5:

> For passive adsorption chemistry, we used different functionalized Pandex paramagnetic particles . . . The functional groups on these particles are amino, dimethylamino, and triethylammonium, or other functional groups that interact strongly with the negative charge of the phosphate moiety of CARD and/or PC. Covalent coupling chemistry, however,

> provides for the immobilization of CARD, PC and CHOL in a form that is more resistant to low concentrations of detergent. The following covalent coupling methods via polar head group and/or via fatty acid moieties can be used for CARD and/or PC: a. SeO2 oxidation. b. PCC (pyridinium chlorochromate) oxidation. c. M-chloroperbenzoic acid oxidation. d. 1,4-Butanediol diglycidyl ether (oxirane) coupling. e. Biotin coupling in the presence of EDC (l-ethyl-3 (3-dimethylamino propyl) carbodilmide). f. Succinic anhydride coupling.

The extensive coupling chemistry taught by WO 91/10138 would not be compatible with proteins and nitrocellulose. For example, covalent coupling by oxidative modification of lipid antigens is disclosed in WO 91/10138 as a preferred coupling method. However, the oxidation reagents required to modify lipid antigens would destroy amino acids sensitive to oxidation, thereby disrupting all or part of the protein antigenic epitope. In addition, WO 91/10138 teaches non-covalent passive adsorption of lipids on the carrier using positively charged groups such as, e.g., amino, dimethylamino, and triethylammonium groups. The positively charged groups on the carrier strongly interact with the negative charge of the lipid phosphate moiety to adhere the lipid to the carrier. The method described in WO 91/10138 is not compatible with all carriers. Nitrocellulose has negatively charged/polarized nitro groups and, therefore, the coupling method is not suitable for use with nitrocellulose carrier material. The manufacturer of nitrocellulose membranes (Schleicher & Schüll, Dassel Germany; now part of General Electric) had no specific data on immobilizing lipids to nitrocellulose in 2002, but generally organic solvents damage nitrocellulose.

10. Likewise, the method of adhering cardiolipin to a substrate disclosed in Pedersen et al., *J. Clin. Microbiol.*, *25*(9): 1711-1716 (1987) is not suitable for use with protein antigens. The Pedersen reference teaches that polyvinyl chloride microtiter plates were coated with an ethanol solution containing VDRL antigen, and the ethanol was evaporated overnight (see page 1712, column 1, paragraph 3). Exposure to ethanol would disrupt protein antigens, rendering a carrier comprising both cardiolipin and a *Treponema*-specific antigen unable to detect anti-*Treponema* antibodies. A scientist in the field would not have been motivated to attempt to bind proteins and lipids together on a single diagnostic carrier faced with the state of the art in 2002.

11. In addition, previous VDRL- and 47 kD *Treponema* antigen-based assays require different test procedures, different storage, and different conservation substances. A typical VDRL antigen-based assay is Arlington Scientific, Inc.'s ASI VDRL Antigen Test, which is a flocculation test for detecting reagin antibodies (Arlington Scientific, Inc.; Springville, UT). The product information sheet for the ASI VDRL Antigen Test is provided as Appendix B (Doc. # 6004-950 NCCLS). The lipid antigen is stored in absolute ethanol and closely sealed to prevent oxidation. The assay requires preparation of an antigen suspension comprising lipid antigens in 1% (w/v) sodium chloride (pH 6.0 +/- 0.1), which must be prepared daily because the aqueous lipid suspension cannot be stored. Formaldehyde also is included in the buffered saline as a preservative. These reagents are not suitable for use with proteins and/or nitrocellulose carriers. Ethanol precipitates protein antigens and destroys nitrocellulose, while formaldehyde can cross-link proteins or disrupt antigenic epitopes.

12. My research team was the first to demonstrate reactivity of VDRL-positive sera with VDRL-antigen bound to nitrocellulose. After extensive experimentation we discovered that the lipid antigen, protein antigen, and buffer influence each other and the sensitivity and specificity of the Treponema test system. Low detergent concentrations resulted in high, unspecific background reaction of the protein antigens. Higher detergent concentrations solubilized lipid antigens from the carrier. It was surprising that cardiolipin antigen reactivity could be maintained at a level required for a diagnostic test while maintaining sensitive and selective reactivity of Treponema protein antigens to anti-Treponema antibodies on the same carrier.

13. I declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

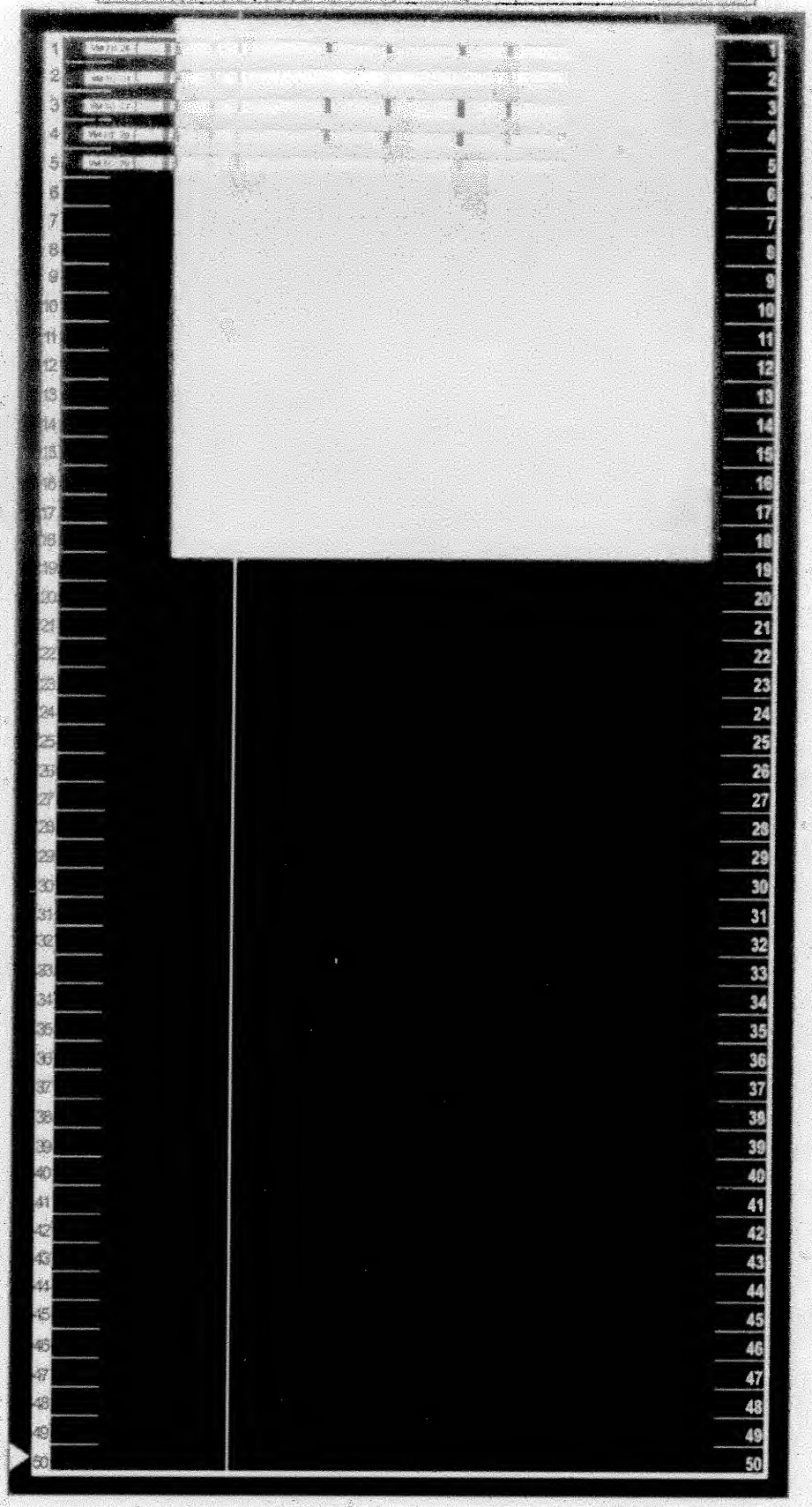
Feb. 4th 2009
Date

Martin Kintrup
Martin Kintrup, Ph.D.

APPENDIX A

ViraStripe®

IgG-Membran + VDRL, geblockt ohne Tween, entw. 0,005 % Tween, anti-IgG, 29.10.02



VM 546, V.+, IgG+, IgM+

VM 1332, V.-, IgG-, IgM-

BD VDRL +

BD VDRL +/-

BD VDRL -

Datum: 29.10.02 Kit Ch.-Bez.-Nr. _____ Antigen Ch.-Bez.-Nr. _____ Temperatur: 23°C Entwicklungszeit: 20' IgG X IgM ___ IgA ___

I: gelöscht auf 'S:\virastripe\Treponema IgM\Experimentelles\Auswertung\Stripes.cdr

APPENDIX B

Subject/Title: ASI VDRL ANTIGEN TEST		Doc#: 6004-950 NCCLS
Effective Date: 11/01	Supersedes Revision/Date: 02/00	Revision: 11/01
Prepared by: ASI	QA Approval by:	Copy/Dept.:

FOR IN VITRO DIAGNOSTIC USE

Cat. No. 950010

1 **INTENDED USE:** The **ASI VDRL Antigen Test** is a qualitative and semiquantitative nontreponemal flocculation test for the detection of reagin antibodies in human serum. These materials are intended to be acquired, possessed and used only by health pr ofessionals.

2 **SUMMARY AND EXPLANATION:** *Treponema pallidum*, the etiological agent of syphilis, induces the production of at least two types of antibodies in human infection: anti-treponemal antibodies that can be detected by FTA-ABS antigen (1), and anti-nontreponemal antibodies (reagin) that can be detected by the VDRL test (2).

3 **PRINCIPLE OF THE PROCEDURE:** The **ASI VDRL Antigen Test** is a microscopic nontreponemal flocculation test to be used for the detection of reagin. The procedure is based on the VDRL antigen being combined at a correct ratio with buffered saline and then mixed with heat-inactivated serum.

4 **REAGENTS**

4.1 ASI VDRL ANTIGEN - 0.03% Cardiolipin and 0.9% Cholesterol in absolute alcohol. Sufficient Lecithin (approximately 0.20 to 0.22%) is added to produce standard reactivity.

4.2 ASI VDRL BUFFERED SALINE - Phosphate-buffered saline, pH 5.9-6.1, containing 0.05% formaldehyde as preservative.

5 **WARNINGS AND PRECAUTIONS**
For *In Vitro* Diagnostic Use

5.1 ASI VDRL ANTIGEN is highly flammable and is irritating to eyes, respiratory system and skin. There is possible risk of irreversible effects and there is risk to an unborn child. Avoid contact with skin and eyes. Do not breathe aerosols. Wear suitable protective clothing. Keep container tightly closed. Keep away from sources of ignition. No smoking. Target organs are blood, intestines, liver, muscle and nervous tissue.

5.2 Observe universal precautions in handling and disposing of the specimens utilized in this test. The CDC/NIH Health Manual "Biosafety in Microbiological and Biomedical Laboratories" describes how these materials should be handled in accordance with Good Laborator y Practice (3).

5.3 Do not pipet by mouth.

5.4 Do not smoke, eat, drink or apply cosmetics in areas where serum samples are handled.

5.5 Any cuts, abrasions or other skin lesions should be suitably protected.

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6 HANDLING AND PROCEDURAL NOTES

6.1 In order to obtain reliable and consistent results, the instructions in the package insert must be strictly followed. Do not modify the handling and storage conditions for reagents or samples.

6.2 Do not use past the expiration date indicated on the kit.

6.3 Do not interchange components of one kit with those of another kit.

6.4 Keep the VDRL ANTIGEN and VDRL BUFFERED SALINE tightly closed at all times to prevent evaporation.

6.5 All glassware, needles and syringes must be clean and dry before use. Rinse all equipment with water, alcohol and acetone in this specific order.

6.6 Do not use glass slides with concavities, wells or glass rings.

7 STORAGE INSTRUCTIONS:

Store VDRL ANTIGEN and BUFFERED SALINE at room temperature (15-30°). The VDRL ANTIGEN should be protected from light.

8 INDICATIONS OF DETERIORATION

8.1 Turbidity or precipitation in controls is indicative of deterioration and the control should not be used.

8.2 Bacterial contamination of reagents or specimens may cause false positive results.

8.3 Any visible discoloration of VDRL ANTIGEN or VDRL BUFFERED SALINE may be indicative of deterioration and the reagent should not be used.

9 SPECIMEN COLLECTION AND STORAGE

9.1 Only serum is suitable for use in this test. Plasma is not acceptable.

9.2 Samples may be maintained in their original tubes at 2-8°C for up to four (4) hours. If longer storage is required, the serum must be separated from the red cells and stored at -15°C or below.

9.3 Frozen samples must be thawed at room temperature before use.

9.4 Samples should be free from bacterial contamination, hemolysis or lipemia.

9.5 If necessary before testing, centrifuge the specimens at a force sufficient to sediment cellular components.

9.6 Samples to be sent out for testing should be placed on ice packs and packaged like any other biohazardous material that could potentially transmit infection.

9.7 With modification, the test procedure can be revised for testing cerebrospinal fluid (CSF) (2).

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10 MATERIALS PROVIDED

VDRL ANTIGEN	2 x 5 ml
VDRL BUFFERED SALINE	2 x 60 ml

11 ADDITIONAL MATERIALS REQUIRED

11.1 Mechanical rotator adjustable to 180±5 rpm and circumscribing 3/4 inch diameter, with humidity cover
11.2 VDRL control sera: reactive, weak reactive, nonreactive
11.3 Saline (0.9% NaCl solution)
11.4 Non-disposable calibrated 18-gauge needle without bevel
11.5 Non-disposable glass syringe, 1 ml or 2 ml
11.6 Bottles, 30 ml, round, narrow-mouth, approximately 35 mm in diameter with ground glass stoppers and flat inner bottom surfaces
11.7 Slides, 2 x 3" with rings approximately 14 mm in diameter. The rings can be paraffin or ceramic, but must be sufficiently high to prevent spillage during rotation.
11.8 Micropipettor, calibrated to deliver 50 µl
11.9 Pipets, glass serological: 1 ml in 1/10 increments, 5 ml in 1/10 increments, 10 ml in 1/10 increments
11.10 Timing device, minute and second capability
11.11 Microscope capable of 100x magnification

12 TEST PROCEDURE

12.1 PREPARATION OF THE VDRL ANTIGEN SUSPENSION

12.1.1 The VDRL ANTIGEN SUSPENSION must be prepared fresh each day.

12.1.2 The temperature of the VDRL BUFFERED SALINE, VDRL ANTIGEN and equipment should be between 23 and 29°C before preparing the suspension. All glassware, pipets and equipment must be dry.

12.1.3 With a 1 ml serological pipet, deliver 0.4 ml of VDRL BUFFERED SALINE to the bottom of a 30 ml round, glass stoppered bottle with flat inner bottom.

12.1.4 Pipet 0.5 ml of VDRL ANTIGEN gradually into the VDRL BUFFERED SALINE while continuously but gently rotating the bottle on a flat surface. Add the VDRL ANTIGEN drop by drop at a rate allowing approximately 6 seconds for 0.5 ml of VDRL ANTIGEN. Keep the pipet tip in the upper third of the bottle. Do not splash the BUFFERED SALINE onto the pipet. The proper rotation speed is obtained when the center of the bottle circumscribes a 2-inch (5 cm) diameter circle approximately three (3) times per second. Expel the last drop of VDRL ANTIGEN from the pipet without touching the pipet to the BUFFERED SALINE, and continue rotation of the bottle for 10 seconds.

12.1.5 Add 4.1 ml of VDRL BUFFERED SALINE from a 5 ml pipet.

12.1.6 Cap the bottle and shake it from bottom to top and back approximately 30 times in 10 seconds. The VDRL ANTIGEN SUSPENSION is ready for use and is usable for 8 hours.

12.1.7 Mix the VDRL ANTIGEN SUSPENSION by gently swirling it each time it is used. Do not mix the SUSPENSION by forcing it back and forth through the syringe and needle since this may cause breakdown of particles and loss of reactivity.

12.1.8 To achieve reliable and reproducible test results, the VDRL ANTIGEN SUSPENSION, controls and test specimens must be at 23-29°C when the tests are performed.

12.2 PREPARATION OF THE SAMPLES

12.2.1 Each serum sample must be heat-inactivated for 30 minutes at 56°C prior to testing.

12.2.2 If heat-inactivation occurs more than four (4) hours prior to testing, reheat the serum for an additional 10 minutes at 56°C.

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12.3 ASSAY PROTOCOL B QUALITATIVE

12.3.1 Using a micropipettor, pipet 50 ml of serum into one 14 mm test circle.

12.3.2 Gently resuspend the VDRL ANTIGEN SUSPENSION.

12.3.3 Draw a sufficient volume of VDRL ANTIGEN SUSPENSION into the needle and glass syringe assembly. Dispense several drops into the 30 ml SUSPENSION bottle to make sure the passage is clear.

12.3.4 Holding the VDRL ANTIGEN SUSPENSION dispensing needle and syringe in a vertical position, dispense exactly one (1) free-falling drop of ANTIGEN SUSPENSION into each circle containing serum.

12.3.5 Place the slide onto the mechanical rotator and cover to maintain humidity. Rotate the slide at 180±5 rpm for four (4) minutes.

12.3.6 Immediately after rotating the slide, remove it from the rotator and read the test microscopically, using 100x magnification. Record the results.

12.4 ASSAY PROTOCOL B SEMIQUANTITATIVE

12.4.1 To quantitate serum samples for determination of endpoint, dilutions can be prepared directly on the glass slide.

12.4.2 Using a micropipettor, dispense 50 µl of saline into the circles numbered 2-4. Do not spread.

12.4.3 Dispense 50 µl of serum onto circles 1 and 2 of the glass slide.

12.4.4 Mix the saline and the serum in circle 2 by drawing the mixture up and down in the pipet 5 or 6 times. Avoid any bubble formation.

12.4.5 Transfer 50 µl from circle 2 to circle 3 and mix as in step (4) above. Repeat this serial dilution procedure to circle 4 and discard 50 µl from the last circle. Circles 1 through 4 represent a dilution series as follows:

Circle:	1	2	3	4
Dilution:	1:1	1:2	1:4	1:8

12.4.6 Gently resuspend the VDRL ANTIGEN SUSPENSION in the 30-ml bottle, and draw sufficient volume into the syringe and needle assembly.

12.4.7 Holding the VDRL ANTIGEN SUSPENSION dispensing needle and syringe in vertical position, dispense several drops into the 30-ml bottle to clear the needle of air. Then add exactly one (1) free-falling drop of ANTIGEN SUSPENSION to each circle.

12.4.8 Place the slide onto the automatic rotator and cover to maintain humidity. Rotate the slide at 180±5 rpm for four (4) minutes.

12.4.9 Immediately after rotation, read the test microscopically using 100x magnification.

12.4.10 If the highest dilution tested (1:8) is reactive, further serial dilutions must be performed until endpoint is observed.

13 **QUALITY CONTROL:** Controls with graded reactivity should be included in each test run to confirm optimal reactivity of the ANTIGEN SUSPENSION. If control samples do not yield the expected response, the assay should be considered invalid and the assay repeated. If the repeat assay does not elicit the expected results for the control

samples, discontinue use of the ANTIGEN SUSPENSION and contact ASI Technical Service.

14 INTERPRETATION OF RESULTS

14.1 INTERPRETATION OF RESULTS- QUALITATIVE

Read and record the results as follows:

Medium to large clumps	Reactive (R)
Small clumps	Weakly Reactive (W)
No clumping or very slight roughness	Nonreactive (N)

14.2 INTERPRETATION OF RESULTS- SEMIQUANTITATIVE

The highest dilution that produces a reactive, not weakly reactive, result is the endpoint titer. In the following example, the titer reported would be 1:2.

TITER	1:1	1:2	1:4	1:8	1:16
RESULT	RR	R	W	N	N

15 LIMITATIONS OF THE PROCEDURE

15.1 Biological false positive reactions occur occasionally with the VDRL test. Such reactions sometimes occur in samples from individuals with a history of drug abuse, or with diseases such as lupus, erythematosus, malaria, vaccinia, mononucleosis, leprosy, viral pneumonia and after smallpox vaccinations.

15.2 Prozone reactions may occur in which reactivity with undiluted serum is inhibited. The prozone phenomenon often gives weakly reactive or "rough" nonreactive results in the qualitative test. Therefore, all specimens with these results must be quantitatively tested until endpoint is reached or until no reactivity is observed.

15.3 In manufacturing, the VDRL ANTIGEN and BUFFERED SALINE are tested only with serum. A modification of the serum test reagents and procedures for CSF testing appears in the "Manual of Tests for Syphilis" (2). The user is responsible for modifying the reagents and procedures and for provision of the required quality control materials according to this manual.

15.4 Pinta, yaws, bejel and other treponemal diseases produce positive reactions in this test (2).

15.5 The results of the **ASI VDRL Antigen Test** must be confirmed by a treponemal test.

15.6 In accord with all diagnostic methods, a final diagnosis should not be made on the result of a single test, but should be based on a correlation of test results with other clinical findings.

16 EXPECTED VALUES

The **ASI VDRL Antigen Test** is evaluated for the equivalence, in its pattern of reactivity, against the CDC Reference VDRL Antigen Suspension.

17 REFERENCES

1. Hunter EF, Deacon WE, Myer PE. 1964. *Public Health Reports*, **79**:410-412.
2. Larsen SA, Hunter EF, Kraus SJ (ed.). 1990. *Manual of Tests for Syphilis*, Public Health Service, Washington, D.C.
3. *Biosafety in Microbiological and Biomedical Laboratories*, 3rd ed. 1993. HHS Publication No. (CDC) 93-8395, Public Health Service, Washington, D.C.